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Influence of Sex and Age on the Activity of Antioxidant Enzymes of Polymorphonuclear Leukocytes in Healthy Subjects

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In this study, the main antioxidant enzymes (AOE) of glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT) and myeloperoxidase (MPO) were identified, and the influence of sex and age in healthy human polymorphonuclear leukocytes (PMNL) was determined. The SOD, GPX, CAT and MPO activities were investigated in intestinal parasite negative human PMNL from 109 healthy subjects aged from 6 to 70 years (55 males and 54 females) using simple and sensitive enzyme assays. Blood cells, such as eosinophils, platelets, neutrophils, monocytes, and macrophages also synthesize antioxidant enzymes (AOE). They constitute an important proportion and are also the major participants in a number of pathological conditions that suggest the involvement of AOE. A linear effect of age on SOD activity ($p<0.05$) both in males and females was found. A similar effect with GPX activity ($p<0.05$) was observed in males only. This showed that the activities of all these enzymes increase with age. In addition, SOD activity was significantly higher in females than males between the age of 19 and 70 years ($p<0.001$). This analysis also showed that there is a negative correlation between the CAT-GPX ($p<0.05$) activities and positive correlations between MPO-GPX ($p<0.05$) activities only in females. No correlation among the other enzyme activities was found in either sex group. This study showed the activities of antioxidant enzyme activities and the correlations of these enyzmes activities with each other in healthy human PMNLs were age- and sex-dependent. This information may assisit in understanding the importance of antioxidant enzymes in the physiological and pathological conditions associated with PMNL.

Key Words: Glutathione peroxidase, superoxide dismutase, catalase, myeloperoxidase, polymorphonuclear leukocytes

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INTRODUCTION

Aerobic organisms possess antioxidant defense systems that deal with the reactive oxygen species produced as a consequence of aerobic respiration. The biological effects of free radicals are controlled enzymatically by a wide range of antioxidant enzymes such as; superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT)[1] *in vivo*. Toxic oxygen plays a role in the aging process.[2] Free radicals are also known to be highly reactive species that have been implicated in the pathogenesis of many diseases. There are several reports, on the SOD, CAT and GPX activities in healthy human erythrocytes, Alzheimer's disease, uremia and cancer.[3] Among these enzymes, SOD catalyzes the dismutation of the superoxide anion (O_2^-) into H_2O_2 ($2O_2 + 2H + H_2O_2 + O_2$), GPX catalyzes the reduction of H_2O_2 to water at the expense of reduced glutathione ($ROOH + 2GSH \quad ROH + H_2O + GSSG$), and CAT detoxifies H_2O_2 and converts the lipid hydroperoxides to nontoxic alcohols ($2H_2O_2 \rightarrow 2H_2O_2 + O_2$). To emphasize the clinical importance of the AOE activities, it can be said that the active O_2^- production and low SOD activity in cancer cells may render the malignant cells to be highly dependent on SOD for survival and be sensitive to SOD

inhibition.[4]

Myeloperoxidase (MPO) is released from the cytoplasmic granules of neutrophils and monocytes by a degranulation process. It reacts with the H_2O_2 formed by a respiratory burst to form a complex that can oxidize a large variety of substances. Among the latter is chloride, which is oxidized initially to hypochlorous acid with the subsequent formation of chlorine and chloramines. These products of the MPO-H_2O_2-chloride system are powerful oxidants, which can have profound biological effects. MPO and H_2O_2 can also be released from a cell where a reaction with chloride can induce damage to the adjacent tissue and therefore, contribute to the pathogenesis of disease. It has been suggested that pulmonary injury, renal glomerular damage, and the initiation of atherosclerotic lesions may be caused by the MPO system.[5]

Blood cells, such as eosinophils, platelets, neutrophils, monocytes, and macrophages also synthesize AOE. Among them, PMNLs constitute an important proportion and are also major participants in a number of pathological conditions that suggest involvement of AOE. Most studies have focused on to whole blood, while studies on PMNL are very limited. There is an increasing understanding the PMNL, AOE and its regulatory role in their function. This study shows the AOE activities in healthy human PMNL according to age-and sex-dependence, and the correlations between each of them. Therefore, this study may point out the discrepancies related to the presence of AOE in PMNL.

It has been reported that, the superoxide anion level generated by PMNL was found to be significantly higher in leukemia patients particularly those with acute lymphocytic and nonlymphocytic leukemia, while the hydrogen peroxide levels were comparable to the control values.[6] Regarding the role and level of the anti-oxidant enzymesin healthy subjects, most studies involved a restricted population and considered only one or two of the main AOE in PMNL. Therefore, the SOD, GPX, CAT and MPO activities were assessed in healthy non-smoking human PMNL by using simple and sensitive enzyme assays, and the influence of sex and age was analyzed.

MATERIALS AND METHODS

Subjects

The SOD, GPX, CAT and MPO activities in human PMNL from 109 healthy subjects aged between 6-70 years (55 males and 54 females) were assayed. None were smokers and none had any known pathologies at the time of sampling. Blood samples were obtained from healthy people who came to the different departments of Erciyes University/Medical Faculty for a regular check-up and students or employees (including their children) of the University. All subjects fasted after midnight prior to blood collection the next morning. In addition, all subjects were examined for intestinal parasites to eliminate the possibility of intestinal parasites affecting the antioxidant enzyme activities.[7] For this, wet mount preparations in 0.9% NaCl, in diluted Lugol's iodine and a flotation technique in a saturated saline solution were used and only the parasite negative subjects were selected.[8]

Preparation of polymorphonuclear leukocytes lysates (PMNL)

Blood samples were collected using a heparinized disposable syringe, and mixed with 6% (w/v) dextran in saline in a ratio of 4:1 and allowed to stand for 1 hour at room temperature. The supernatant was centrifuged at 275 × g for 10 min and removed. The pellet was resuspended in an 8 ml Krebs Ringer phosphate buffer (KRPB), layered over 3 ml of Histopaque-1077(Sigma Chemical Co., St. Louis,Mo, USA) spun at 450 × g for 30 min at 37℃ and the pellet containing PMNL was obtained. The residual erythrocytes were lysed by adding 9 volumes of distilled water to this pellet, followed by vigorous shaking for 30 sec. The osmolarity was restored by adding 1 volume of 9% (w/v) NaCl. The cell suspension was centrifuged at 275 × g for 5 min and the supernatant was discarded. The pellet was washed 3 times with KRPB and resuspended in 2 ml KRPB. The cell numbers in the final suspension were counted and the suspension stored at -80℃. The purity of the PMNL population was

approximately 90% (the PMNL suspension was frozen at -20℃ and thawed six times) and homogenized using an ultrasonicator. The protein content of the homogenate was measured using the method reported by Lowry et al.[9]

Determination of SOD activity

The SOD enzymatic activity was measured according to the method reported by Sun et al.[10] It is based on the inhibition of nitroblue tetrazolium (NBT) reduction with xanthine-xanthine oxidase used as a superoxide generator. One unit of SOD activity was defined as the amount of protein that inhibits the rate of NBT reduction by 50%. The enzyme activity was expressed as U/mg protein.

Determination of CAT activity

The CAT activity was measured by the method reported by Lück.[11] One unit of CAT activity was defined as the amount of enzyme, which liberated half of the peroxide oxygen from H_2O_2 solution in 100 s at 25℃. The enzyme activity was expressed as U/mg protein.

Determination of GPX activity

GPX activity was determined according to Paglia and Valentine[12] using hydrogen peroxide as substrate. The reaction mixture contained 2.48 ml of a 50 mM/l phosphate buffer, pH 7 (Sigma); 0.01 mL 112.5 mM/l sodium azide; and 4.6 U glutathione reductase (Type III, Sigma). The reaction was initiated by adding 0.1 ml 2.2 mM H_2O_2 to the reaction mixture containing 500-1000 μg protein. The change in the optical density was read at 340 nm for 4 min. The data is expressed as nmol NAPH oxidized to NADP/mg protein/min by using the molar extinction coefficient of 6.200 at 340 nm. All data was corrected for the linear NADPH oxidation by H_2O_2 alone in the absence of the enzyme protein, which was always < 5% of the total reaction.[13]

Determination of MPO activity

MPO activity was measured according to the Klebanoff et al.[14] The assay mixture 0.3 ml 100 mM phosphate buffer (pH 6.0; 0.3 ml of 10 mM H_2O_2, 0.5 ml of 20 mM o-dionisidine (freshly prepared) in deionized water; and 10 μl of the PMNL homogenate in a final volume of 3.0 ml. The absorbance at 460 nm was measured for 100 min. All measurements were carried out in duplicate. One unit of MPO was defined as that degrading 1 μmol H_2O_2/min at 25℃; the specific activity is given as U/mg of protein. A molar extinction coefficient of 11.300 for the oxidized o-dianisidine was used for the calculation.

Statistical analysis

All values are expressed as a mean ± S.D. The results were analyzed using a student t-test, and linear regression analysis. A p value < 0.05 was considered statistically significant.

RESULTS

Infulence of age and sex on enzyme activities

Table 1 shows there is a linear effect on SOD activity according to age in both females (r^2: 0.471, $p<0.05$, Fig. 1) and males (r^2:0.3084, $p<0.05$, Fig. 2), and also on GPX activity in females only (r^2: 0.3831, $p<0.05$, Fig. 3). This suggests that the activity of all these enzymes increases with age. In addition, although no differences in SOD activity were observed among males and females between 6 - 18 years of age ($p>0.05$), the SOD values were observed to be significantly higher in females than in males between the age of 19 - 70 ($p<0.001$) (Table 1). A significant effect of the age

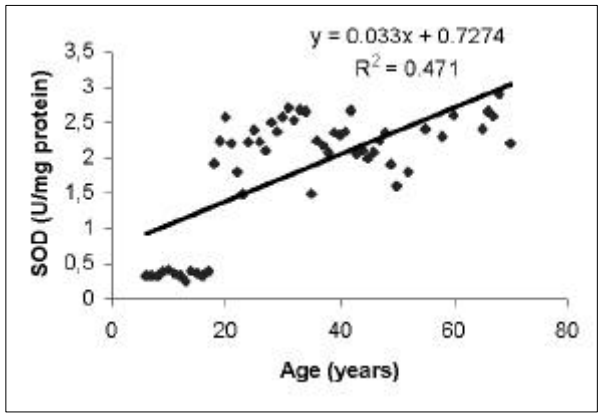

Fig. 1. Correlation between age and SOD activity in female human PMNL.

of donors on CAT and MPO activities ($p>0.05$) was not found both in females and males (results not shown) and in GPX activity in males ($p>0.05$) (results not shown).

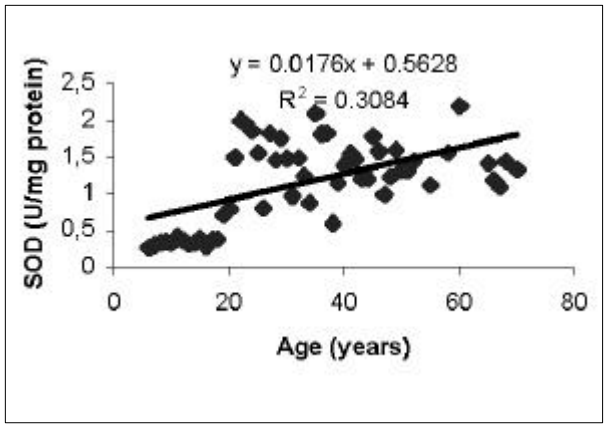

Fig. 2. Correlation between age and SOD male human PMNL.

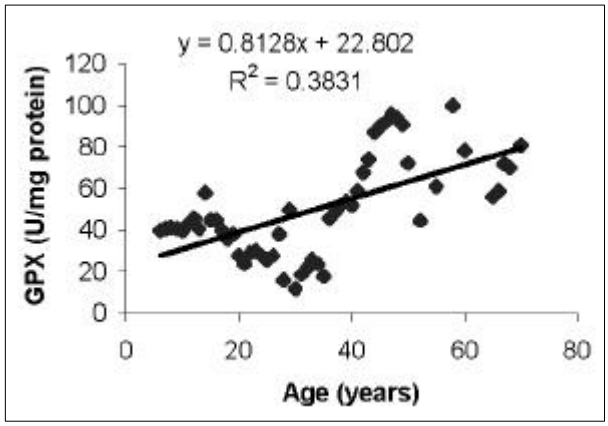

Fig. 3. Correlation between age and GPX activity in female human PMNL.

Comparison of relationship between enzyme activities

When the relationship between the enzyme activities were assessed, a negative correlation was found between CAT-GPX (r^2: 0.1512, $p<0.05$, Fig. 4), and a positive correlation between MPO-GPX (r^2: 0.3162, $p<0.05$, Fig. 5) activities in females only.

No significant correlations were found among the CAT-GPX ($p>0.05$), MPO-GPX ($p>0.05$) activities in males only, and the CAT-SOD ($p>0.05$), CAT-MPO ($p>0.05$), MPO-SOD ($p>0.05$) and GPX-SOD ($p>0.05$) activities both in females and males (results not shown).

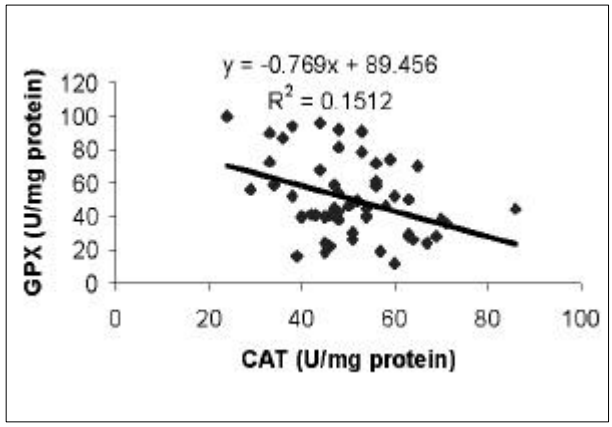

Fig. 4. Correlation between CAT and GPX activities in female human PMNL.

Table 1. Comparison of the Enzyme Activities in PMNL

	CAT		MPO		GPX		SOD	
	M (n=55)	F (n=54)	M (n=55)	F (n=54)	M (n=55)	F (n=54)	M (n=55)	F (n=54)
Age (yr)								
12±3 (6-18)	51 ± 8	51 ± 7	366 ± 55	317 ± 28	50 ± 7	45 ± 5	0.36 ± 0.05	0.39 ± 0.07
40±14 (19-70)	62 ± 26	58 ± 17	338 ± 1	339 ± 98	47 ± 20	44 ± 24	1.50 ± 0.39	2.32 ± 0.37
p value	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p<0.05$	$p<0.05$	$p<0.05$
	NS	NS	NS	NS	NS			

CAT, catalase; MPO, myeloperoxidase; GPX, glutathione peroxidase; SOD, superoxide dismutase; values (U/mg protein) are mean± SD; n, number of subjects; M, male; F, female.

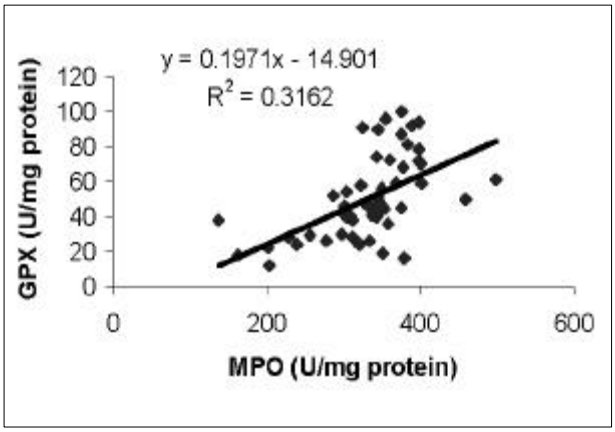

Fig. 5. Correlation between MPO and GPX activities in female human PMNL.

DISCUSSION

Considering healthy subjects, the reported measurements of the oxidative enzymes in the PMNL studies involved only a small population sample with only one or two of the AOE activities being measured in individuals. A complete understanding of the AOE system in human blood as a function of sex and age that can influence AOE activity requires the measurement of all the primary AOEs. In this report, the activities of all the main AOEs, i.e., SOD, CAT, GPX and MPO were measured in PMNL from healthy subjects and the influence of sex and age was analyzed. A linear positive effect of age on SOD activity was found in both in males and females, showing that the activities of these enzymes in PMNL increases with age.

SOD catalyzes the dismutation of O_2^- to H_2O_2.[15] The activity of this enzyme was found to increase in human PMNL with age both in males and females. This age-dependent increase appears to contrast with other results obtained from human blood.[15,16] This increase according to age may be due partly to the origin of the isolated PMNL samples used. This enzyme is one of the antiradical activity enzymes. This enzyme is known to depend on the Zn and Cu concentrations. Its catalytic activity depends on the presence of a prosthetic group containing Cu, Zn stabilizes the apoenzyme in the native configuration.

In addition, although no differences were observed among females and males between age of 6-18, males have a lower SOD activity level compared to females between 19-70 years of age. Although the causes of this variability are unclear, the possibility that these sex-dependent differences may be in one part be related to the hormones, especially sex hormones, was examined. The reason for this is that between the ages of 6-18, sex hormones are in the progress of development, and between 19-70 the hormones have reached the onset of maturity and work effectively and efficiently.

A linear positive effect of age on GPX activity was found only in females. Although we were unable to detect a similar kind of a significant effect of age on the GPX activity in males, there still appears to be a positive correlation between them.

GPX is the key enzyme in the glutathione redox cycle, which responsible for the reduction of hydroperoxides. It contains 4 selenium (Se) atoms bound as the selenocystein moieties that confer its catalytic activity. GPX has an absolute requirement for reduced glutathione as a co-substrate, which undergoes inactivation by hydroxyl radicals (OH) and O_2.[17] On the other hand, the reason that GPX was exhibited the highest variability are at present time unknown. However, the results are in agreement with data reported by Bolzan, Blum and Lux.[17-19]

In this study, no sex- or age-dependent correlation between the CAT and the MPO activities were found in healthy human PMNL. This suggests that the CAT and MPO activities are not dependent on sex or age. Unfortunately, the reason why the activity of one of the AOEs (SOD) is sex- and age-dependent, and one of them (GPX) is just age and partly sex-dependent is unclear. It is obvious that hormone related studies with all the enzyme activities are needed to the make the above assumptions clear.

In this study, a negative correlation between CAT and GPX was observed in females. This could be partly due to capacity of both enzymes to eliminate intracellular H_2O_2.[20,21] It is also known that both GPX and CAT provide a defense system against free radical injury.[22,23] This finding is in agreement with the theory that a lower GPX level may increase H_2O_2 to concentrations that inactivate SOD. This is because H_2O_2 can also be

formed spontaneously to a considerable degree if O_2^- production increases.[23]

On the other hand, a positive correlation between MPO-GPX activities was also found in females only. It is known that GPX catalyzes H_2O_2 reduction to water at the expense of reduced glutathione. MPO also interacts with the H_2O_2 generated by the respiratory burst, and duration of O_2^- generation during the respiratory burst is regulated by MPO.[24] Therefore, it is most likely that the positive correlation between MPO-GPX and age deal with the H_2O_2 level.

In this study, the other enzyme activities in PMNL had a tendency to either increase or decrease and had positive or negative correlations with age- or sex-. However, the trend was not statistically significant: [Negative correlations were observed among; CAT-MPO (in females and males), CAT-GPX (in males), MPO-SOD (in females and males) and GPX-SOD (in females and males), and positive correlations were observed among SOD-CAT (in females and males), MPO-GPX (in males)].

This variability may be at least in part, hormone dependent. In addition, weight, blood pressure, menopause and the intake of some drugs (e.g. anti-inflammatory agents, anti-depressants and thyroid hormones) can modify the AOE activities. Accordingly, it is suggested that for clinical purposes, the involvement of hormone related studies involved in further studies might be necessary.

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